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## **The prion protein knockout mouse: a phenotype under challenge**

Steele, A D ; Lindquist, S ; Aguzzi, A

**Abstract:** The key pathogenic event in prion disease involves misfolding and aggregation of the cellular prion protein (PrP). Beyond this fundamental observation, the mechanism by which PrP misfolding in neurons leads to injury and death remains enigmatic. Prion toxicity may come about by perverting the normal function of PrP. If so, understanding the normal function of PrP may help to elucidate the molecular mechanism of prion disease. Ablation of the Prnp gene, which encodes PrP, was instrumental for determining that the continuous production of PrP is essential for replicating prion infectivity. Since the structure of PrP has not provided any hints to its possible function, and there is no obvious phenotype in PrP KO mice, studies of PrP function have often relied on intuition and serendipity. Here, we enumerate the multitude of phenotypes described in PrP deficient mice, many of which manifest themselves only upon physiological challenge. We discuss the pleiotropic phenotypes of PrP deficient mice in relation to the possible normal function of PrP. The critical question remains open: which of these phenotypes are primary effects of PrP deletion and what do they tell us about the function of PrP?

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## Review

# The Prion Protein Knockout Mouse

## A Phenotype Under Challenge

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### KEY WORDS

transmissible spongiform encephalopathy, amyloid, PrP

### ABBREVIATIONS

PrP	prion protein
KO	knockout
GPI	glycosylphosphatidylinositol
Cu	copper
CC	charge cluster
HC	hydrophobic core
SOD	superoxide dismutase

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### ABSTRACT

The key pathogenic event in prion disease involves misfolding and aggregation of the cellular prion protein (PrP). Beyond this fundamental observation, the mechanism by which PrP misfolding in neurons leads to injury and death remains enigmatic. Prion toxicity may come about by perverting the normal function of PrP. If so, understanding the normal function of PrP may help to elucidate the molecular mechanism of prion disease. Ablation of the *Prnp* gene, which encodes PrP, was instrumental for determining that the continuous production of PrP is essential for replicating prion infectivity. Since the structure of PrP has not provided any hints to its possible function, and there is no obvious phenotype in PrP KO mice, studies of PrP function have often relied on intuition and serendipity. Here, we enumerate the multitude of phenotypes described in PrP deficient mice, many of which manifest themselves only upon physiological challenge. We discuss the pleiotropic phenotypes of PrP deficient mice in relation to the possible normal function of PrP. The critical question remains open: which of these phenotypes are primary effects of PrP deletion and what do they tell us about the function of PrP?

### INTRODUCTION

The prion protein (PrP<sup>C</sup>) is a conserved glycoprotein tethered to cell membranes by a glycosylphosphatidylinositol (GPI) anchor.<sup>1</sup> PrP<sup>C</sup> denotes “cellular” or “normal” PrP to differentiate it from PrP<sup>Sc</sup> for “scrapie” or disease associated isoform of PrP<sup>C</sup>. PrP<sup>C</sup> is expressed in many tissues, most abundantly in brain, heart, muscle, and also in select lymphoid and myeloid cells.<sup>2</sup> The role of PrP<sup>Sc</sup> in the pathogenesis of the transmissible spongiform encephalopathies (TSE), the prion diseases, has been intensively studied.<sup>1-5</sup> Conversely, much less attention has been focused on the role of PrP<sup>C</sup> in normal physiology.<sup>6</sup> Of note, normal function studies of proteins associated with other neurodegenerative diseases, such as amyloid precursor protein and the secretases for Alzheimer’s disease,<sup>7</sup>  $\alpha$ -synuclein for Parkinson’s disease,<sup>8</sup> and huntingtin for Huntington’s disease,<sup>9</sup> are helping to provide deeper insights into the pathophysiology of these diseases. Analogously, a clearer understanding of the function of PrP<sup>C</sup> in homeostasis may provide valuable insights into the molecular pathways of prion pathogenesis. However, the extent of overlap between understanding the pathogenic dysfunction of PrP<sup>Sc</sup> in prion diseases and the normal function of PrP<sup>C</sup> in cell physiology remains to be determined (depicted in Fig. 1).

Many approaches have been utilized to understand the physiological function of PrP<sup>C</sup>, including but not limited to the identification of multiple interaction partners, human genetic studies of the *Prnp* (prion protein gene) locus, ectopic and overexpression of PrP<sup>C</sup> in a variety of cell types and organisms, and finally deletion or ‘knockout’ (KO) studies in the mouse,<sup>10</sup> cow,<sup>11</sup> and even goat,<sup>12</sup> providing additional exciting tools for understanding aspects of PrP<sup>C</sup> physiology that may not be addressable in mice (Fig. 2). The search for protein interaction partners of PrP<sup>C</sup>, by a variety of methods, has led to interesting candidates but functional demonstration of the importance of these interactions is still missing.<sup>2,13</sup> Furthermore, overexpression and ectopic expression studies of PrP<sup>C</sup>, or expression of mammalian PrP<sup>C</sup> in lower organisms is yet to reveal an irrefutable function for PrP<sup>C</sup>. Another approach includes large scale genetic association studies to look for *PRNP* mutations or polymorphisms associated with human genetic disorders. Some interesting genetic associations with Alzheimer’s disease susceptibility have been found but lack consensus in the field.<sup>14-16</sup> Polymorphisms in PrP<sup>C</sup> have been associated with rare forms of cortical malformation,<sup>17</sup> differences in surgical outcome for a form of epilepsy,<sup>18</sup> and even learning and memory.<sup>19</sup>

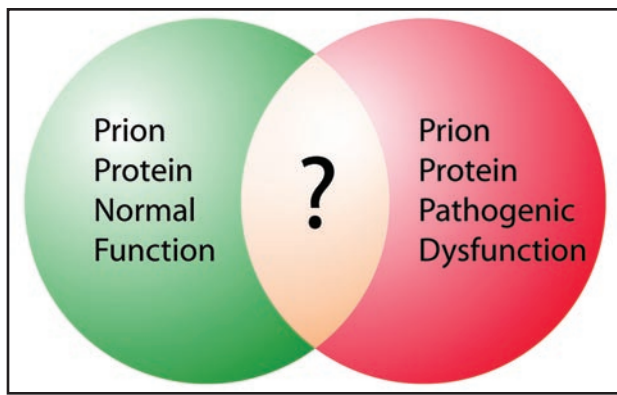


Figure 1. The overlap between the normal function of PrP<sup>C</sup> and the pathogenic dysfunction of PrP<sup>Sc</sup> in disease depicted as a Venn diagram. The extent of overlap between the normal function of PrP<sup>C</sup> and its role in prion disease is open to speculation, the circles could have a much greater or perhaps even less overlap.

A large pool of *PRNP* mutations have been identified in humans, but all of these are associated with familial prion diseases, or appear to represent harmless polymorphisms.<sup>20</sup> Perhaps there are heretofore undiscovered humans with small chromosomal deletions that encompass the *PRNP* locus, or even mutations that render humans haploinsufficient or even null for PrP. Such human patients may tell us volumes about the function of PrP<sup>C</sup> and perhaps that it is dispensable for normal life as is the case for mice<sup>10</sup> and cows.<sup>11</sup>

Because PrP<sup>C</sup> is so conserved among mammals, there was great expectation that ablation of *Prnp* in the mouse would reveal a normal function for this enigmatic gene.<sup>21</sup> Since the PrP KO mice have no overt phenotype,<sup>10,22</sup> it was clear that PrP<sup>C</sup> is not essential for the survival of the laboratory mouse. However, genetic compensation and developmental plasticity may mask the phenotype of PrP<sup>C</sup> deficient mice and thus, it may take an appropriate challenge to reveal any phenotype. Although the original reports on the *Zurich* and *Edinburgh* PrP KO mice (two different targeting strategies to delete PrP, named after the city where the experiments took place) reported “no phenotype”, many subsequent studies have revealed that this KO mouse has an abundance of phenotypes, some of which have been contested and many of which are subtle (Table 1). This review summarizes the recent research in determining the normal function of PrP<sup>C</sup> by utilizing the PrP KO mouse. Although many claims to PrP<sup>C</sup> function have been generated from the study of cultured cells,<sup>3,23</sup> we will mostly confine our discussion to studies utilizing mice: the PrP KO, the deletion of the PrP<sup>C</sup> homolog doppel (Dpl), and overexpression transgenics of PrP<sup>C</sup> and Dpl. *Prnp* is one of the most frequently knocked out mammalian genes, and a plethora of PrP-deficient mice have been generated with a wealth of strategies. Detailed reviews of the construction of the various available PrP KO mice exists.<sup>24,25</sup> The neurodegeneration caused by the ectopic expression of Dpl in the *Nagasaki* and *Rcm* PrP KO (generated with a different gene targeting strategy than the *Zurich* and *Edinburgh* PrP KOs) has been reviewed in detail,<sup>24,25</sup> and will be discussed only in so far as PrP<sup>C</sup> and Dpl function(s) are concerned.

## THE CAVEATS OF A PHENOTYPE

Given the abundance of phenotypes attributed to the deletion of PrP, we will first discuss several technical concerns relating to

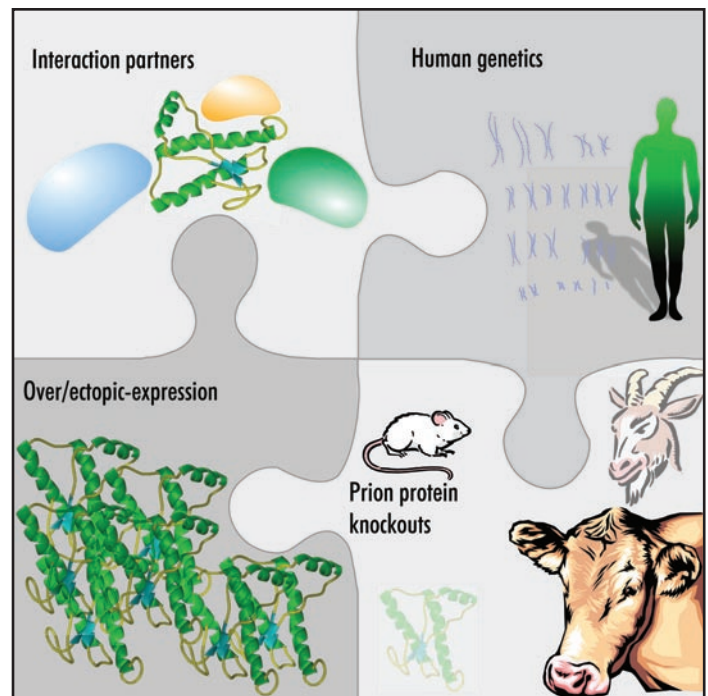


Figure 2. Different approaches to study PrP<sup>C</sup>'s normal function. There are many ways to approach the study of the normal function of PrP<sup>C</sup>, none of which have conclusively demonstrated PrP<sup>C</sup>'s function. Interacting partners of PrP<sup>C</sup> have yielded many interesting candidates, human genetic studies have found associations of PrP<sup>C</sup> with diseases beyond prion disease and even to learning and memory, over- and ectopic-expression studies constitute another approach to determine the function of PrP<sup>C</sup>, and finally, the focus of this review, the PrP KO has given some clues to the function of PrP<sup>C</sup>.

KO studies and potential caveats of phenotypes in PrP KO mice. The advent of “gene-targeting”, or the specific deletion/replacement of chromosomal segments, in mice has revolutionized functional studies of mammalian genes in vivo. However, technical aspects of generating gene-targeted mice from embryonic stem cells can create a potential caveat to interpreting phenotypic data. This caveat arises because often the resultant mice utilized for functional studies are maintained on “mixed” genetic backgrounds, a random mixture of alleles from embryonic stem cells (often a 129 sub-strain) and the parental line (C57Bl/6, Balb/c, or others). Three concerns arise when working on mice of a mixed genetic background, which encompass most studies of PrP KO mice: 1) increases in the phenotypic variability and thus a higher chance of spurious results 2) the heterozygosity at many loci increases the noise of most measurements, potentially obscuring subtle phenotypes, and 3) the possibility that alleles linked to the deleted gene (which is continually selected for by investigators during breeding) are actually responsible for the phenotype in question.<sup>26</sup> Another point to consider is that depending on how the investigator maintains the KO line (either by inbreeding separate populations of PrP<sup>-/-</sup> and PrP<sup>+/-</sup>, or by intercrossing PrP<sup>+/-</sup> mice) we also have to contend with genetic drift, that is the tendency for certain alleles to become “fixed” (100% frequency) in populations simply by chance.

The availability of the fully-sequenced mouse genome, in concert with physical maps of polymorphic short-tandem repeats, would allow for eliminating the confounding genetic factors enumerated above. For example, the “speed congenic” technologies which are being offered commercially—but can be enacted in-house by

real-time PCR and sequencing—enable selective breeding of mice that are genetically homogeneous at any given chromosomal region. However, almost none of the phenotypes described in the following pages have been ascertained using such genetic controls—and this represents a major caveat in the interpretation of phenotypic data. Of the various PrP KO mice that have been generated,<sup>25</sup> only the *Edinburgh* KO has been maintained on a pure 129/Ola background<sup>22</sup> and this strain background is notoriously difficult to work with because of poor breeding and several other abnormalities.<sup>26</sup> Backcrossing PrP KO mice onto a pure genetic background helps to eliminate this concern. However, despite intensive backcrossing, regions on the chromosome adjacent to the *Prnp* locus are carried along through all the generations of backcrossing, such that even after 12 generations of backcross the KO allele would be flanked by about 1% of the ES cell genome.<sup>26</sup> As a consequence, it is difficult to conclude with certainty that a phenotype is due to the genetic background of the mouse or the deleted gene in question. Another point worth considering is that on pure backgrounds it is possible to obtain a phenotype that is a combination of the deletion in question (i.e., PrP KO) and a specific allele(s) of the background (i.e., a C57Bl/6 allele of a gene). Such a KO and strain background synergistic phenotype would be difficult to replicate unless other investigators are using the same mice.

Rescue experiments, whereby a transgene is reintroduced into the KO to rescue a phenotype, are time-consuming and are not “fool-proof”, but represent one approach toward eliminating an effect of a linked allele or a chance observation in a mixed genetic background. On the other hand, such reliance on rescue experiments led to the erroneous assignment of pathological phenotypes to the deficiency of PrP (in *Nagasaki* PrP KO mice),<sup>27</sup> whereas the phenotype was in reality brought about by the overexpression of Dpl in a PrP KO background.<sup>25</sup>

A separate concern from genetic background arises from intensive and multi-faceted hunting for phenotypes. Since all biological measurements, but particularly those made on animals, are associated with intrinsic variability, we expect to find some spurious phenotypes just by chance. For example, if the null hypothesis is that PrP KO mice are not different from wild-type with a conventional threshold of significance of  $p < 0.05$ , we expect that if 100 labs study the PrP KO that as many as five may find spurious phenotypes. This is an example of the “multiple hypothesis testing problem” and is an inescapable reality of biological research. Independent confirmation of findings in more than one type of the PrP KO mouse, for example the *Edinburgh* or *Zurich* PrP KO mice, or by more than one laboratory will be important for building confidence in PrP KO phenotypes.

## THE CLEAREST OF PrP PHENOTYPES: PrP KNOCKOUT MICE ARE RESISTANT TO PRION INFECTION AND CANNOT REPLICATE PRIONS

To date, the clearest phenotype of the PrP KO is resistance to infection with prions.<sup>28,29</sup> This experiment—originally designed to disprove the prion hypothesis<sup>21</sup>—solidified its central tenet: the requirement of a host protein for prion replication. Further variations of this experiment have taught us that PrP<sup>C</sup> expression is required for prion-induced toxicity.<sup>30</sup> Neurografting brain tissue from wild-type mice into PrP KO brains revealed that *Prnp*<sup>+/-</sup> tissue grafts replicated prions with accompanying damage to neurons while nearby PrP-deficient tissue was unharmed.<sup>31</sup> Thus, PrP<sup>C</sup> on

neuronal cells is required for prion propagation associated toxicity. This evidence supports our conjecture that deciphering the normal function or signaling pathway through which PrP<sup>C</sup> operates will help illuminate the devastating sequence of events in prion disease.

The PrP KO mouse enabled another important series of studies that defined some of the sequence elements required for PrP<sup>Sc</sup> to retain infectivity by expressing truncated PrP transgenes on the KO background and infecting these mice with prions. This approach revealed that the octapeptide repeats (Fig. 3) were not necessary for prion replication or toxicity, but indicated that they may be required for the rampant spongiosis and production of high titers of prion infectivity normally associated with prion diseases.<sup>32,33</sup>

## PrP IN SLEEP REGULATION

Sleep disturbances and altered circadian rhythms were the first documented phenotypes in PrP KO mice,<sup>34</sup> other than the resistance to prion infection which is technically a lack of a phenotype.<sup>28</sup> PrP<sup>C</sup> might be involved in regulating sleep, as certain mutations in *PRNP* cause a prion disease known as fatal familial insomnia.<sup>35</sup> This disease eventually results in broadly disseminated neurodegeneration but one key symptom is a nearly complete inability to sleep. Sleep deficits are also a documented feature in human Creutzfeldt-Jakob disease of sporadic origin.<sup>36</sup> Tobler and colleagues found that during a normal light/dark cycle PrP KO mice had similar patterns of running wheel activity as controls. However, in constant darkness, wild-type mice display a shorter circadian period (as is customary for wild-type mice without circadian cues) whereas PrP KO mice remarkably maintain a normal period as if still “entrained” by light.<sup>34</sup> This finding was shown in both the *Edinburgh* and *Zurich* PrP KO mice. Further studies by Tobler and colleagues revealed that PrP KO mice have more fragmented sleep episodes than do controls, leading the authors to conclude that PrP<sup>C</sup> plays a role in promoting sleep continuity.<sup>37</sup> The fact that PrP<sup>C</sup> alters sleep and that PrP<sup>Sc</sup> production also leads to sleep abnormalities supports our hypothesis that understanding PrP<sup>C</sup> function will help to understand prion disease. But in nearly a decade since this phenotype was documented there is still no clarity as to how PrP<sup>C</sup> regulates sleep at a molecular level.

## A ROLE FOR PrP IN OXIDATIVE STRESS: COPPER BINDING, SOD-ACTIVITY AND MITOCHONDRIA

A considerable amount of work has focused on the copper (Cu) binding and potential anti-oxidant function of PrP<sup>C</sup>. The genesis of this work is the observation that recombinant PrP<sup>C</sup> binds to Cu and that copper levels were diminished in brains of PrP KO mice.<sup>38</sup> The ability of PrP<sup>C</sup> to bind Cu has been well supported but alteration in Cu content in PrP KO brains is controversial.<sup>39</sup> A study by Wong et al. suggests that PrP<sup>C</sup> is involved in defense against oxidative damage.<sup>40</sup> They observed higher levels of oxidized proteins and lipids in the brains of *Edinburgh* PrP KO mice.<sup>40</sup> A similar situation was observed in *Zurich* PrP KO mice by Klamt and colleagues, who further found that superoxide dismutase (SOD) activity was significantly decreased in the brain and muscle of PrP KO mice.<sup>41</sup> On the other hand, Waggoner et al. could not detect differences in enzymatic activity of Cu-Zn superoxide dismutase.<sup>39</sup> This may be due to differences in experimental conditions.<sup>42</sup> However, mouse genetic experiments argue against a SOD-like activity for PrP<sup>C</sup> in vivo.<sup>43</sup> We reasoned



that if PrP<sup>C</sup> has a SOD activity, then deficiency in both PrP<sup>C</sup> and SOD1 will result in diminished SOD activity compared to deficiency in SOD1 only, and conversely PrP<sup>C</sup> overexpression in a SOD1 KO or WT background will result in increased SOD activity. However, these genetically defined crosses did not reveal any alterations in SOD activity with respect to PrP<sup>C</sup> deletion.<sup>43</sup>

One intriguing phenotype at the cellular level is the reduction in number of mitochondria per cell in the CA1 region of the hippocampus and in the myocardium of *Edinburgh* PrP KO.<sup>44</sup> The mice used in this study were inbred 129/Ola strain, which reduces the likelihood of genetic background artifacts but could also result in strain specific effect of a gene deletion (discussed above). This study was stimulated by the identification of differentially expressed genes in the PrP KO, revealing only three genes that were differentially expressed—all of which were involved in mitochondrial biogenesis and physiology.<sup>44</sup> Also, mitochondria having morphological abnormalities were more abundant in the PrP KO (*Edinburgh*).<sup>44</sup> It is noteworthy that we identified swollen mitochondria in a transgenic model of prion disease, where PrP is mislocalized to the cytosol, another possible connection between PrP<sup>C</sup> in normal physiology and disease.<sup>45</sup> Lobao-Soares and colleagues sought to extend the observation of abnormal mitochondria in PrP KO to a functional level by measuring mitochondrial respiration in several regions of PrP KO brains, but found similar levels between PrP KO and controls in all regions examined.<sup>46</sup> However, it should be noted that isolated mitochondria were utilized in this study and it is possible that small numbers of abnormal mitochondria may not be detected by their assays. Further studies will be required to clarify the role of PrP<sup>C</sup>—if any—in oxidative stress and mitochondrial physiology.

## THE ROLE OF PrP IN THE IMMUNE SYSTEM, PHAGOCYTOSIS AND AS A MICROBIAL RECEPTOR

Recent reports have suggested a role for PrP<sup>C</sup> in cellular internalization pathways, perhaps a function that has been co-opted by microbes. Work from Rafael Linden and colleagues add to the growing list of phenotypes for the PrP KO. *Prnp* null macrophages displayed increased rates of phagocytosis in vitro and in vivo, leading to the conclusion that under physiological conditions PrP<sup>C</sup> negatively regulates phagocytosis.<sup>47</sup> This is extremely surprising since the expression levels of PrP<sup>C</sup> in macrophages are typically below the limits of detection (Christina Sigurdson and A. Aguzzi, unpublished data). Studies of microbial pathogenesis in PrP KO may be connected with a role for PrP<sup>C</sup> in phagocytosis or cytokine production. Watarai and colleagues have discovered that the PrP KO is more resistant to infection with the bacterial pathogen *Brucella abortus*.<sup>48,49</sup> Localization and biochemical experiments pinpointed bacterial heat shock protein 60 as an interaction partner with PrP<sup>C</sup> on the cell surface.<sup>48</sup> It would be interesting to examine the consequences of PrP<sup>C</sup> overexpression and *B. abortus* infection. These results were not confirmed in a different laboratory in studies using *B. suis*.<sup>50</sup>

Virologists have made use of the PrP KO as well, revealing yet another phenotype likely relating to cellular internalization pathways. In this study, Thackray and Bujdoso demonstrate that PrP KO are refractory to infection with a neurotropic herpes simplex virus whereas PrP overexpression transgenics (Tga20) were highly susceptible to infection.<sup>51</sup> Studies of viral titers and maturation markers suggest that viral replication is retarded in PrP KO in favor of establishing latency.

Intriguingly, the viral infection induced neuronal cell death much more dramatically in PrP overexpression transgenics, connecting PrP<sup>C</sup> to cell survival pathways (discussed below). Several of these findings were confirmed in a follow-up study by the same group.<sup>52</sup>

Recent work suggests that PrP<sup>C</sup> may play a role in the immunological synapse. Ballerini et al., demonstrated that PrP<sup>C</sup> is important in an interaction between T cells and dendritic cell.<sup>53</sup> PrP<sup>C</sup> was dispensable on T cells for this interaction but PrP<sup>C</sup> on dendritic cells was important in stimulating T cells in vitro and in vivo assay.<sup>53</sup> Another study reports that  $\alpha\beta$  T cells are greatly diminished in *tga20* PrP overexpression transgenic mice, and these mice also display an atrophy of the thymus.<sup>54</sup> However, this phenotype may represent an insertional mutagenesis artifact since *tga19* transgenic mice derived from the same construct do not show these anomalies (AA, unpublished data). One study has attempted to link copper uptake and interleukin expression in T cells, finding a slight delay in interleukin-2 expression in PrP KO T cells.<sup>55</sup> To summarize, PrP<sup>C</sup> may be important for host-pathogen interactions, immune synapses and T cell homeostasis, but further studies will be needed to decipher the role of PrP<sup>C</sup> in the immune system.

## NEURONAL EXCITABILITY

The high level of PrP<sup>C</sup> expression in neuronal cells led to an interest in detecting electrophysiological defects in the PrP KO. This topic is no less controversial than any other we have discussed in this review, but the weight of the evidence clearly lies on the side of altered neuronal excitability in PrP KO neurons. Electrophysiological studies of PrP KO were first under-taken by John Collinge and John Jefferys, who found that CA1 neurons in *Zurich* PrP KO had faster after-hyperpolarization currents and were impaired in long term potentiation (LTP).<sup>56</sup> Jean Manson and colleagues had similar findings in purebred *Edinburgh* PrP KO.<sup>57</sup> In addition it was shown that both wild-type and a familial mutant human PrP<sup>C</sup> were capable of rescuing this electrophysiological phenotype when expressed as transgenes in the PrP KO background.<sup>58,59</sup> Soon after these findings were reported opposing reports surfaced. Herms et al. examined synaptic transmission in Purkinje cells of *Zurich* PrP KO but did not detect any differences from controls.<sup>60</sup> Another group found no differences between *Zurich* PrP KO and controls in the CA1 region of the hippocampus.<sup>61</sup> Over the ensuing years there have been several other attempts to clarify the electrophysiological phenotype (or lack thereof) in PrP KO.<sup>62-64</sup> However, only one thing is clear—detection of the electrophysiological phenotype depends on which line of mice is being used, who is investigating, and the age of the PrP KO being used.<sup>65</sup> It is worth noting that authors on two of the papers reporting “no phenotype”<sup>60,61</sup> have reversed their position in later studies.<sup>66,67</sup> Finally, the post natal neuronal-specific KO of PrP<sup>C</sup> showed a reduction of after-hyperpolarization potentials in neurons in CA1,<sup>68</sup> an identical phenotype to what had been originally reported by Collinge and colleagues.<sup>56</sup>

The neuronal excitability phenotypes may relate to one of the strongest phenotypes of PrP KO, which presents under the challenge of seizure inducing drugs. *Zurich* PrP KO are much more susceptible to repeated doses of pentylenetetrazol and kainic acid, both of which induce seizures.<sup>69</sup> Approximately 50% of PrP KO died from a single administration of kainic acid while 100% of control animals survived.<sup>69</sup> This result has been confirmed independently

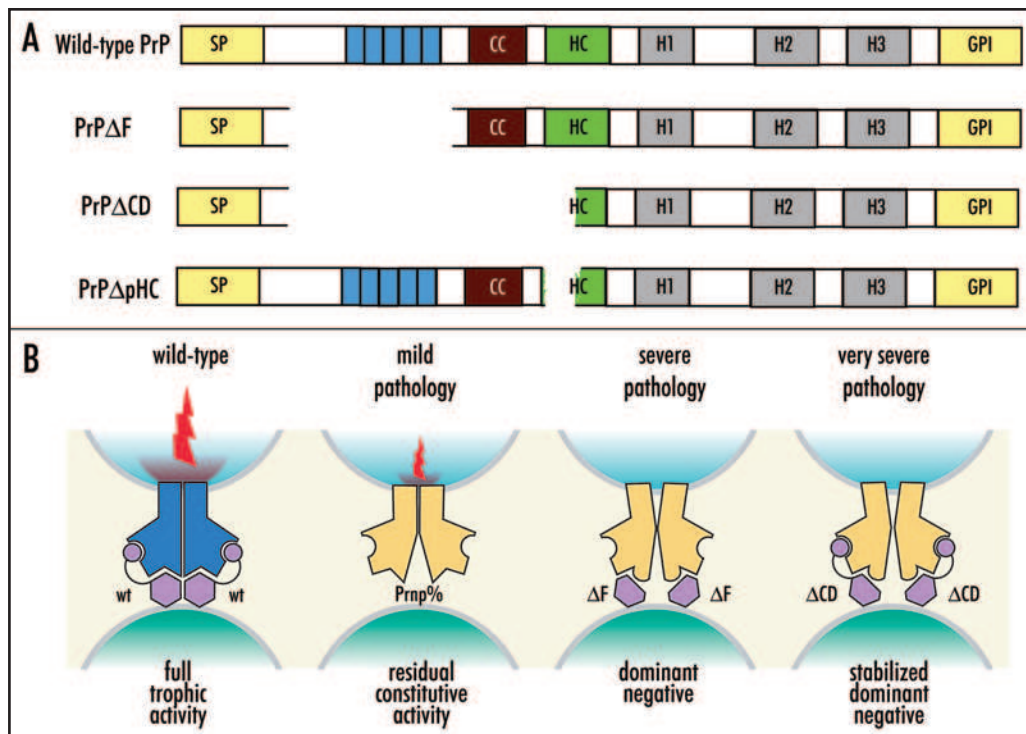


Figure 3. A model for the effects of PrP<sup>C</sup> deletion and deletion mutants of PrP<sup>C</sup>. (A) Schematic diagram of wild-type PrP<sup>C</sup> and deletion mutants. SP, signal peptide; octapeptide repeats are indicated in blue; CC, charge cluster; HC, hydrophobic core; H1, H2, H3 Helix 1, 2 and 3, respectively; GPI, GPI-anchor addition sequence (B). PrP (black) consists of a globular C-terminal domain (hexagon) and a N-terminal flexible tail (arch) encompassing the octapeptide repeats (ORs) (circle). The model rests on the following assumptions: (1) PrP activates a hitherto unidentified receptor (PrP<sub>R</sub>) which transmits myelin maintenance signals (flashes); (2) in the absence of PrP, PrP<sub>R</sub> exerts some residual activity, either constitutively or by recruiting a surrogate ligand; (3) the activity of PrP and its mutants requires homo- or heterodimerization, and induces dimerization of PrP<sub>R</sub>; and (4) PrP dimers containing PrP $\Delta$ CD or PrP $\Delta$ CD trap PrP<sub>R</sub> in an inactive dominant-negative state. Finally, (5) the OR region stabilizes the interaction between PrP and PrP<sub>R</sub>, but does not contribute directly to signaling.

by Rangel, et al., who also note increased neuronal cell death in PrP KO mice injected with kainic acid.<sup>70</sup> The increased seizure sensitivity may be due to higher levels of ectonucleotidase activity which destroys adenosine, an endogenous anticonvulsant agent, in PrP KO mice.<sup>71,72</sup> Finally, it is also worth noting that a defect in neuronal architecture of the hippocampus in *Zurich* PrP deficient mice has been reported and may be relevant to several of the findings discussed above.<sup>73</sup> Timm stained sections of the hippocampus from PrP KO had more sprouting of axons than did controls in the granule cell layer of the dentate gyrus and the infrapyramidal region of CA3 region.<sup>73</sup> This is said to resemble the mossy fiber collateral and terminal sprouting seen in certain human epilepsies.

## BEHAVIORAL PHENOTYPES: IS PrP INVOLVED IN LEARNING AND MEMORY?

If they bear any relevance to real life, the electrophysiological defects described above for the PrP<sup>C</sup> null neurons might manifest in the behavior of the PrP KO mouse. The abundant expression of PrP<sup>C</sup> in regions important in learning and memory, such as the hippocampus, has lead to a series of behavioral studies aimed at detecting abnormalities in PrP KO mice. Initial studies by Charles Weissmann, Hans Peter Lipp, and colleagues did not detect any phenotype of *Zurich* PrP KO mice in a long-term study using maze tests.<sup>10,74</sup> Further, a study by Roesler et al. failed to detect any abnormalities in anxiety nor inhibitory avoidance learning in PrP KO mice.<sup>75</sup> Cognitive defects have been detected in PrP KO mice by Criado

and colleagues who found that spatial learning was defective in PrP KO mice.<sup>62</sup> This phenotype was PrP-dependent as it was rescued by crossing PrP KO mice with a transgene driving expression of hamster PrP<sup>C</sup> under a neuron-specific promoter.<sup>62</sup> Another study describes PrP KO mice as having normal short- and long-term memory at three months of age but impairments by 9 months of age.<sup>76</sup> Aged PrP KO mice also showed less exploratory activity in an open field.<sup>76</sup> A follow-up study suggested that the interaction of PrP<sup>C</sup> and laminin may be key to memory consolidation in rats,<sup>77</sup> although there is no clarity about which molecular events might be triggered by the binding of PrP<sup>C</sup> to laminin. Finally, a study in humans suggests that the M129V polymorphism in PrP<sup>C</sup>, which influences susceptibility to prion infection in humans-may be involved in learning and memory.<sup>19</sup>

In an attempt to reveal a behavioral phenotype, investigators have challenged PrP KO mice in various ways during phenotypic testing. Coitinho et al. dosed PrP KO mice with various psychotropic drugs and interestingly, PrP KO mice show a decreased response to the psychotropic drug MK-801, which normally causes increased motor activity.<sup>78</sup> Amphetamine and caffeine induced hyper-locomotion to an equal extent in PrP KO mice and controls.<sup>78</sup> Nico et al. subjected *Zurich* PrP KO mice to acute stress by foot shock or swimming trial and found that PrP KO mice showed less anxiety than controls after these treatments.<sup>79</sup> In non-stress conditions, PrP KO mice appeared identical to controls.<sup>79</sup> Another study notes a very subtle increase in locomotor activity in PrP KO mice in an open field test.<sup>75</sup> This increased locomotor activity has not been observed using extensively backcrossed C57Bl/6 PrP KO mice (both *Edinburgh* and *Zurich*) in the home cage using high

resolution techniques recently used to study prion disease in detail,<sup>80</sup> however, our testing conditions are not equivalent to an open field test (ADS and SL, unpublished results).

## DIVERSE NEUROPROTECTIVE PROPERTIES OF PrP

Many studies have claimed that protection against neuronal damage is one of PrP<sup>C</sup>'s *raison d'être*. Neuroprotection (defined generically as protecting neurons from dysfunction or death) may represent one of the best-supported functions of PrP<sup>C</sup>. This protection applies to both physiological challenges and to a peculiar yet fascinating paradigm whereby the closest homolog of PrP<sup>C</sup>, Dpl, when ectopically expressed in the brain causes loss of Purkinje neurons in the cerebellum but only in a PrP KO background.<sup>27</sup>

We will begin our discussion of PrP's protective properties with one of the most agreed upon observations—PrP KO mice are much more susceptible to ischemic damage. McLennan and colleagues were the first to document that PrP KO mice are more susceptible to stroke.<sup>81</sup> They were led to the PrP KO through studying a dramatic upregulation of PrP expression at sites of stroke in human brains.<sup>81</sup> Subsequent studies have replicated and extended these results in acute<sup>82</sup> and long term models of ischemia<sup>83</sup> and even in transgenic rats.<sup>84</sup> Interestingly, transgenic overexpression of PrP in the mouse does not protect above wild-type PrP levels<sup>85</sup> while in the rat increasing PrP levels did confer protection. Weise and colleagues note that PrP KO mice have lower levels of phosphorylated-Akt both in basal conditions and during ischemic injury, pointing towards a general role of PrP in activation of cell survival pathways.<sup>83</sup> Other researchers have noted significant increases in the phosphorylation of ERK-1 and -2, STAT-1, and JNK-1 in ischemic PrP KO brains.<sup>85</sup> Recently, Gains and colleagues have extended PrP's neuroprotective spectrum. They dosed neonatal PrP KO mice and controls with a high dose of ethanol, a paradigm for inducing Bax mediated apoptosis, and noted a dramatic increase in cell death in brains of PrP KO mice.<sup>86</sup> Another brief report documents an enhanced brain injury in PrP KO mice after head trauma,<sup>87</sup> however, it is likely that these mice overexpress Dpl and therefore display a confounding effect. To test PrP's neuroprotective function in another setting, we crossed PrP KO mice to several transgenic models of neurodegenerative disease—Huntington's, Parkinson's and Alzheimer's disease. Much to our surprise, the phenotypes of these diseases were largely unaltered by PrP deletion (ADS, Z. Zhou, W. Jackson, M. Moskowitz, S. Lindquist, unpublished). Thus, the wide-ranging neuroprotective functions of PrP have limitations and these observations of protection in unique models need to be understood in mechanistic detail.

The second well-studied paradigm in which PrP exerts a protective function deals with the neurotoxicity induced by its nearest homolog. The exciting and circuitous discovery of Dpl began with conflicting reports on the phenotype of the PrP KO, with two groups reporting no phenotype<sup>10,22</sup> and one group reporting a late onset ataxia and Purkinje cell loss in *Nagasaki* PrP KO mice.<sup>27,88</sup> Later it was determined by several groups that a previously undescribed tightly linked homolog of PrP<sup>C</sup> (called "doppel" for "Downstream of the *Prnp* locus") was upregulated by the deletion strategy used in the *Nagasaki* PrP KO line. The *Nagasaki* deletion strategy fused PrP's promoter to Dpl, driving expression of Dpl in the brain, where it is not normally expressed.<sup>89,90</sup> Subsequent experiments determined that the toxicity induced by ectopic Dpl expression in the PrP KO is

abrogated by reintroduction of a single copy of PrP<sup>C</sup>.<sup>91,92</sup> However, both Weissmann and Katamine have shown that PrP<sup>C</sup> cannot suppress higher amounts of transgene driven Dpl expression,<sup>93,94</sup> suggesting that Dpl and PrP<sup>C</sup> are competitive antagonists and can be stoichiometrically titrated against each other. Interestingly, PrP<sup>C</sup> devoid of the octapeptide repeats (amino acids 23–88) is incapable of rescuing Dpl toxicity, suggesting an important role for the N-proximal region of PrP<sup>C</sup> in its neuroprotective functions.<sup>95</sup> However, a trafficking defect in this deletion mutant of PrP was not ruled out, so PrP may not be reaching the cell surface in these transgenic mice.

A similar phenomenon is observed when an artificial deletion mutant of PrP<sup>C</sup>, missing amino acids 32–134 and termed "ΔF", is expressed as a transgene in the PrP KO. This transgenic mouse develops a dramatic loss of granular neurons in the cerebellum, referred to as "Shmerling syndrome" (Fig. 3a).<sup>96</sup> The deletions of amino acids 32–121 or 32–134, but not shorter deletions of the N-terminus, caused this phenotype. The "ΔF" truncated PrP<sup>C</sup> targeted specifically to Purkinje cells causes these cells to die in a PrP KO background showing that this is a cell-autonomous phenomenon akin to Dpl toxicity.<sup>97</sup> It has also become clear that truncated PrP<sup>C</sup> and Dpl are toxic to myelinated cells in a PrP KO background.<sup>98</sup> The white matter pathology was detected in the cerebellum, brainstem, and spinal cord in "ΔF" mice and extended more broadly in Dpl overexpression where it was also found in the forebrain, pyramidal projections, and the corpus callosum.<sup>98</sup> Further deletion mapping of PrP<sup>C</sup> has narrowed the critical region to forty amino acids in the middle of PrP, 94–134. When PrP lacking these amino acids is expressed in PrP KO mice it results in a severe motor phenotype brought about by extensive central and peripheral myelin degeneration (Fig. 3A).<sup>99</sup> PrP<sup>C</sup> lacking the octapeptide repeats was able to rescue this deletion mutant induced phenotype unlike the case of Dpl toxicity discussed above.

Most interestingly, careful histopathological analysis clearly identified a myelin degeneration phenotype in PrP KO mice.<sup>91,99</sup> Since this phenotype is seen both in *Zurich 1* and in *Nagasaki* mice, it cannot result from spurious overexpression of Dpl and may indeed represent a consequence of PrP deficiency. The similarity of this phenotype to Shmerling's syndrome is striking—although Shmerling's syndrome is much more severe and is visible in much younger mice. This finding suggests that myelin maintenance may represent an important physiological function of PrP<sup>C</sup>, and that the defects seen in Shmerling's disease may represent an exaggerated form of a PrP<sup>C</sup> deficiency syndrome.

In the studies discussed above, all toxic mutants displayed disruption of the charge cluster (CC, residues 95–110) and a part of the hydrophobic core (HC, residues 111–121) of PrP (Fig. 3). Toxicity was ameliorated by co-expressing PrP variants with intact CC and HC, even if these variants lacked the octapeptide region. We therefore posit that PrP exerts its neuroprotective activity by signalling through the central domain to an unknown receptor (tentatively termed PrP<sub>R</sub>). In all paradigms investigated, the phenotype was determined by the stoichiometry of mutant to full-length PrP, suggesting that PrP, any of the various PrP mutants, and PrP<sub>R</sub> form hetero-oligomeric complexes (Fig. 3B). The mild pathological phenotype of PrP KO mice suggests that myelin integrity is supported by residual PrP<sub>R</sub> activity, whereas disruption of the central domain (CD) domain sequesters PrP<sub>R</sub> in a dominant-negative state. Complex

Table 1 **Proposed functions for PrP from analysis of PrP knockout mice**

Phenotype in PrP KO	Reference	Confirmed?	Opposing?
<b>Circadian/Sleep regulation</b>			
Abnormal circadian rhythm	34		
Interrupted sleep patterns	37		
<b>Oxidative stress: copper binding, SOD-activity and mitochondria</b>			
Decreased copper content in brain	38		39
Decreased SOD activity; increased oxidative damage	40, 42	41	39, 43, 46
Reduction/abnormal mitochondria	44		46
<b>Immune system, phagocytosis and as a microbial receptor</b>			
Increased phagocytosis	47		
Resistance to infection with <i>B. abortus</i>	48		50
Resistance to infection with HSV-1	51	52	
Immune synapse, T cell response	53		
Interleukin expression	55		
<b>Neuronal excitability</b>			
Impaired long term potentiation	56	62, 59, 67, 64, 66, 57, 58	60, 61, 63, 65
Increased susceptibility to seizures	69	70	
Mossy fiber disorganization in hippocampus	73		
<b>Behavioral phenotypes</b>			
Cognitive defects/memory impairment	76, 62		10
Increased locomotor / exploratory activity	75	76	
Increased hyperlocomotion induced by MK-801	78		
Decreased anxiety	79		
<b>Neuroprotection</b>			
Susceptible to Dpl toxicity	27	89–92	
Susceptible to “ΔF” PrP induced toxicity	96	97, 98	
Enhanced susceptibility to ischemia	81	82,83,85	
Enhanced susceptibility to ethanol induced apoptosis	86		
Enhanced susceptibility to traumatic brain injury	87		
<b>Stem/precursor cells</b>			
Impaired self-renewal of hematopoietic stem cells	100		
Decreased proliferation of neural precursors	102		
<b>Miscellaneous</b>			
Abnormality in dentin in teeth	109		

stability could be influenced by domains distinct from those involved in executing signal transduction: the context-dependent toxicity of PrP missing part of the hydrophobic core (PrP<sub>ΔPHC</sub>) implicates the octapeptide repeat as one such domain.<sup>99</sup> Although deletion of 40 amino acids produced a powerfully toxic molecule, ablation of eight amino acids within this domain (PrP<sub>ΔPHC</sub>) was innocuous to both wild-type and PrP KO mice. Crossing experiments show that PrP<sub>ΔPHC</sub> is not functionally equivalent to PrP. The toxicity of PrP<sub>ΔF</sub> was diminished, yet that of PrP<sub>ΔCD</sub> was augmented by coexpression of PrP<sub>ΔPHC</sub>. In the frame of the signaling model, the deletion in PrP<sub>ΔPHC</sub> may affect the interaction between PrP and PrP<sub>R</sub>. Verification of the model presented above requires the physical identification of PrP<sub>R</sub>. Towards that goal, it will be crucial to identify the cellular constituents, which may not necessarily all consist of protein, binding differentially to PrP<sup>C</sup>.

## A ROLE FOR PrP IN STEM/PROGENITOR CELL BIOLOGY

Recently, it was demonstrated that PrP<sup>C</sup> is expressed on the surface of hematopoietic stem cells.<sup>100</sup> Zhang and colleagues then challenged PrP KO bone marrow with serial transplantations into lethally irradiated recipient mice. After several transplantations, the repopulating potential of PrP KO bone marrow was exhausted whereas control bone marrow was still competent to repopulate lethally irradiated recipient mice, demonstrating that PrP KO hematopoietic stem cells were deficient in “self-renewal”.<sup>100</sup> Reintroduction of PrP into PrP null bone marrow cells rescued this defect in self-renewal, arguing against an artifact of genetic background. The molecular pathway by which PrP promotes the self-renewal of hematopoietic stem cells remains unclear but is consistent with many of the suggested protective functions of PrP<sup>C</sup>. The studies of PrP<sup>C</sup> in hematopoietic



stem cells prompted an examination of PrP<sup>C</sup> expression/function in neural stem/precursor cells in the adult brain. Adult neurogenesis is normally neuroanatomically restricted to the dentate gyrus of the hippocampus and the subventricular zone.<sup>101</sup> We pulse labeled BrdU into PrP KO and overexpression transgenics and noted that in the dentate gyrus PrP KO mice had a lower level of proliferating cells whereas in the PrP<sup>C</sup> overexpression transgenic cell proliferation was enhanced in the subventricular zone.<sup>102</sup> Culturing of neural progenitor cells from embryonic PrP KO mice and overexpression transgenics revealed that PrP<sup>C</sup> may promote the exit from a precursor state and maturation into a neuronal lineage.<sup>102</sup> The signaling pathway through which PrP<sup>C</sup> presumably operates in this process is still undetermined. Collectively, these results demonstrate that careful scrutiny reveals a subtle function for PrP<sup>C</sup> in stem and precursor cell biology and it will be interesting to examine whether PrP<sup>C</sup> functions in other adult stem cell populations that can be more readily isolated. In concert with the possible protection against seizure, ischemia, pathogen infection, and evolutionary conservation of PrP<sup>C</sup>, these results add to the evidence for its relevance to mammalian physiology.

## **FUTURE PROSPECTS FOR DETERMINING PrP FUNCTION FROM IN VIVO STUDIES: BEYOND THE PRION PROTEIN KNOCKOUT MOUSE**

Several investigators have expressed PrP<sup>C</sup> in yeast in order to better understand the disease-associated properties of this protein.<sup>103</sup> Furthermore PrP<sup>C</sup> has been expressed in yeast (which do not express a PrP homolog) to investigate normal function, but perhaps this is aiming too “low” in terms of model organisms as initial studies have not revealed any role for PrP<sup>C</sup> in copper transport, a well characterized process in yeast.<sup>104</sup> Interestingly PrP<sup>C</sup> expression rescues Bax induced cell death in yeast;<sup>105</sup> however the significance and relevance of this paradigm is unclear even in mammalian cells.<sup>106</sup> Given the remarkable conservation of PrP structure among vertebrates,<sup>107,108</sup> the use of non-mammalian models could open up new avenues for prion research. For instance, the first dramatic phenotypes of PrP loss- and gain-of-function have been produced in zebrafish (Edward Málaga-Trillo, Gonzalo Solis, Yvonne Schrock, Lydia Luncz, Venus Thomanetz, and Claudia Stuermer, personal communication). Notably, early knockdown of PrP in fish embryos is lethal but can be partially rescued by expression of mouse PrP, ruling out potential off target effects of the knockdown construct. Analysis of the molecular pathways involved in fish PrP function should guide future studies in the mammalian system. Studies in the rat offer the advantage of larger brains that make surgical and other interventions more feasible.<sup>77</sup> However, it is unlikely that deletion of PrP<sup>C</sup> will be much more informative in the rat than it has been for the mouse. Very recently it has become possible to study PrP<sup>C</sup> function in ruminants lacking PrP<sup>C</sup> which appear normal up to 20 months of age.<sup>11</sup> Also, hemizygous PrP<sup>C</sup> deletion goats now exist and homozygous null animals should be forthcoming.<sup>12</sup> Perhaps the longer lifespans of the cow and goat will reveal age dependent phenotypes associated with PrP<sup>C</sup> deletion. Likewise it may be worthwhile to delete *Prnp* in a non-human primate, where much more detailed cognitive testing could be conducted. That being said, it remains possible that the PrP KO mouse holds the key to understanding PrP<sup>C</sup> function, perhaps even through the study of unexpected phenotypes, such as the recently reported tooth abnormality in PrP KO mice.<sup>109</sup>

Looking to PrP<sup>C</sup>'s closest homologue in the mouse, Dpl, may be fruitful for determining the function of PrP<sup>C</sup>. Interestingly, the Dpl KO results in male sterility,<sup>110,111</sup> a phenotype that can be rescued by testis specific expression of a PrP with an N-terminal deletion in the testes suggesting a functional equivalence of Dpl and the C terminus of PrP (A. Aguzzi, unpublished results). Deletion of both PrP<sup>C</sup> and Dpl (dKO) had no discernable phenotypes aside from the male infertility present in Dpl KO mice.<sup>112,113</sup> Deciphering the pathway through which Dpl affects male fertility could inform future studies of PrP<sup>C</sup> function. A more distant cousin of PrP, called “Shadoo”<sup>114</sup> may also hold promise for revealing PrP<sup>C</sup>'s function.

Will the transgenic mice expressing PrP<sup>C</sup> without a GPI anchor (GPI-) prove useful for elucidating the normal or the disease function of PrP? These mice are very intriguing because replication of prion infectivity is more or less unaffected whereas toxicity is significantly suppressed in the PrP KO background.<sup>115,116</sup> It may be that the hypothesized signal transduction properties of PrP<sup>C</sup> are defective without a GPI anchor attachment, leaving the GPI-mice fully competent for PrP<sup>C</sup>'s only ironclad function—prion replication. This mouse provides an excellent tool in distinguishing PrP<sup>Sc</sup> replication from toxicity.

There are many unresolved questions with respect to PrP<sup>C</sup> function: What is the connection between PrP function and pathogenesis? What is the functional relevance of differential expression of PrP<sup>C</sup> in subpopulations of neurons in the brain? How do we assign the many described phenotypes in PrP KO mice to discrete molecular pathways? Perhaps physiological stress will only bring out the effects of non-relevant linked genes, leading down a garden path of genetic artifacts? Is the conformational diversity attained by PrP<sup>Sc</sup> reflecting a possible conformational diversity of PrP<sup>C</sup> as recently speculated by Stanley Prusiner?<sup>117</sup> It is fascinating to speculate that this labile structure of PrP<sup>C</sup> may encipher its function. Hopefully one day the question of PrP<sup>C</sup> function will be settled, but for now there remains plenty of work to do!

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